REMARKS

Applicant wishes to thank Examiner Romeo for the courtesy extended to Nancy Vensko, attorney of record, on July 21, 2005. The Interview Summary Form PTOL-413A summarizes the discussion held at the personal interview. The present response to the outstanding Office Action includes the substance of the Examiner Interview.

A. Disposition of Claims

Claims 19, 55, and 56 are pending in this application. Claim 19 has been amended and Claims 55 and 56 have been added to describe with more particularity the claimed subject matter and thus for reasons unrelated to patentability. Support for the amendment is found throughout the patent specification, for example, at p. 9, line 27 to p. 10, line 17. Additionally, the benefit claim has been amended to make reference to prior nonprovisional applications per 37 CFR 1.78(a)(2)(i) that includes, besides identification of, relationship between applications. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

B. Compliance with 35 USC 112/1 enablement

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the enablement requirement. Under MPEP 2164, the test for enablement is whether one skilled in the art could make or use the subject matter defined by the claims without undue experimentation. Further under MPEP 2164.01(a), the Wands factors are to be considered in determining whether any necessary experimentation is undue. Here, the specification is enabling with respect to the claimed methods because Frzb induces bone.

i) First, there is considerable direction and guidance in the specification with respect to the claimed methods related to Frzb inducing bone. As proof, attached is the post-filing date art of Chung et al., 2004, J Bone Miner Res 19: 1395. Chung et al. 2004, a third party publication authored by investigators unrelated to the inventors, describes the contribution of the specification published as Hoang et al., 1996, J Biol Chem 271: 26131 as follows:

> sFRP3 [i.e., Frzb] was originally isolated from bovine articular cartilage and was implicated in bone development because of its bone- and cartilage-inducing activities.

(sFRP3 is Frzb, as evidenced by Chang et al., 1999, Hum Mol Genet 8: 575, attached, at Table 1.) The present application was published as Hoang et al. 1996 and Wang et al., 1997, Cell 88: 757, both of record, but reattached for the convenience of the Examiner. The quotation shows that Chung et al. 2004, at p. 1396, col. 1, last ¶, credits Hoang et al. 1996 as being the first to establish that Frzb induces bone. Because Hoang et al. 1996 published this contribution of the specification, the conclusion is that the specification teaches that Frzb induces bone. A review of the patent specification confirms this conclusion.

As described in the patent specification, articular cartilage extracts were prepared to characterize protein fractions with *in vivo* chondrogenic activity (Example 1). Trypsin digestion of highly purified chondrogenic protein fractions allowed the identification of several unique peptides by amino acid sequencing (Example 2). The inventors discovered a novel cDNA encoding a deduced 36-kDa protein by using degenerate oligonucleotide primers derived from a 30-residue peptide in reverse transcription polymerase chain reactions (Example 3). Its N-terminal domain showed ~50% amino acid identity to the corresponding region of the *Drosophila* gene *frizzled*, which has been implicated in the specification of hair polarity during development (Id.). Hydropathy and structural analyses of the open reading frame revealed the presence of a signal peptide and a hydrophobic domain followed by multiple potential serine/threonine phosphorylation sites and a serine-rich C terminus (*Id.*). Cell fractionation studies of primary bovine articular chondrocytes and transfected COS cells suggested that the protein is membrane-associated (Example 5). *In situ* hybridization and immunostaining (Example 4) of human embryonic sections demonstrated predominant expression surrounding the chondrifying bone primordia and subsequently in the chondrocytes of the epiphyses in a graded distribution that decreased toward the primary ossification center (Example 6). Transcripts were present in the craniofacial structures but not in the vertebral bodies (*Id.*). Because it is expressed primarily in the cartilaginous cores of developing long bones during embryonic and fetal

development and is homologous to the polarity-determining gene *frizzled*, the authors reasoned that this gene, which they named *frzb,* is involved in morphogenesis of the mammalian skeleton.

In sum, the contribution of the specification published as Hoang et al. 1996 established that Frzb induces bone.

ii) Second, there was a high level of skill in the art at the time the application was filed. The level of skill in the DNA cloning art was that of a postdoctoral fellow working in the laboratory. *Amgen Inc. v. Hoechst Marion Roussel Inc.*, 57 USPQ2d 1449, 1518 (D. Mass. 2001). Thus, the level of skill in the art was high.

iii) Third, all of the methods needed to practice the invention were well known. As of the 11 October 1996 priority date, for guidance regarding such conditions, see, for example, Sambrook, et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and Wiley & Sons, Inc., New York.

iv) Per MPEP 2164.01(a), the In re Wands Court held that the specification was enabling with respect to the claims at issue and found that "there was considerable direction and guidance" in the specification; there was "a high level of skill in the art at the time the application was filed;" and "all of the methods needed to practice the invention were well known." Similarly, here, as indicated above, there was considerable direction and guidance in the specification; there was a high level of skill in the art at the time the application was filed; and all of the methods needed to practice the invention were well known.

Westendorf et al., 2004, Gene 341: 19, cited by the Examiner, is not contradictory. Although some Wnt inhibitors have negative impacts on bone formation (Westendorf et al. 2004, p. 26, col. 2, ¶ 3.3.2), and Frzb is known to antagonize some Wnts (e.g.., contribution of specification published as Wang et al. 1997), Chung et al., 2004 (*supra*) confirms that Frzb induces bone. Chung et al. 2004, at Abstract ("We found that sFRP3 unexpectedly increased osteoblast differentiation"). *Id.* at p. 1398, entire ¶ entitled "sFRP3 promotes osteoblast differentiation". *Id.* at Discussion, p. 1399 – 1400 ("To our surprise, in this study, we discovered that sFRP3 treatment promoted osteoblast differentiation. Because of the known effect of Wnts to promote osteoblast differentiation and bone formation, we predicted that sFRP3 treatment would antagonize Wnt effects and cause an inhibition of differentiated functions of osteoblasts.

However, this proved not to be the case, as shown by several lines of experimental evidence. First, sFRP3 treatment increased ALP activity in a dose-dependent manner [] ... Second, sFRP3 treatment increased osteocalcin levels in the conditioned medium of osteoblasts. ... Third, the number of mineralized nodules was significantly increased by sFRP3 treatment. ... Thus, we have convincing experimental data that sFRP3 is a promoter of differentiated functions of osteoblasts in vitro.) Chung et al. 2004 hypothesizes that the osteoblastic differentiation reported in this paper are mediated by a mechanism apart from modulation of the Wnt/catenin axis (at p. 1401, col. 1, last sentence), thus supporting the prediction in the inventors' Cell paper that Frzb may have another mode of action in addition to inhibition of Wnt signaling (Wang et al. 1997, p. 764, col. 1, first full ¶, last sentence). Put together, Chung et al. 2004 corroborates by additional experimentation the contribution of the specification published as Hoang et al. 1996 that Frzb induces bone.

Consequently, here, considering all the factors related to the enablement issue, it must be concluded that it would *not* require undue experimentation to practice the claimed methods because Frzb induces bone.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

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Dated: 8/25/05

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Primary Structure and Tissue Distribution of FRZB, a Novel Protein Related to *Drosophila* Frizzled, Suggest a Role in Skeletal Morphogenesis*

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Articular cartilage extracts were prepared to characterize protein fractions with *in vivo* chondrogenic activity (Chang, S., Hoang, B., Thomas, J. T., Vukicevic, S., Luyten, F. P., Ryba, N. J. P., Kozak, C. A., Reddi, A. H., and Moos, M. (1994) *J. Biol. Chem.* 269, 28227–28234). Trypsin digestion of highly purified chondrogenic protein fractions allowed the identification of several unique peptides by amino acid sequencing. We discovered a novel cDNA encoding a deduced 36-kDa protein by using degenerate oligonucleotide primers derived from a 30-residue peptide in reverse transcription polymerase chain reactions. Its N-terminal domain showed ~50% amino acid identity to the corresponding region of the *Drosophila* gene *frizzled*, which has been implicated in the specification of hair polarity during development. Hydropathy and structural analyses of the open reading frame revealed the presence of a signal peptide and a hydrophobic domain followed by multiple potential serine/threonine phosphorylation sites and a serine-rich C terminus. Cell fractionation studies of primary bovine articular chondrocytes and transfected COS cells suggested that the protein is membrane-associated. *In situ* hybridization and immunostaining of human embryonic sections demonstrated predominant expression surrounding the chondrifying bone primordia and subsequently in the chondrocytes of the epiphyses in a graded distribution that decreased toward the primary ossification center. Transcripts were present in the craniofacial structures but not in the vertebral bodies. Because it is expressed primarily in the cartilaginous cores of developing long bones during embryonic and fetal development (6–13 weeks) and is homologous to the polarity-determining gene *frizzled*, we believe that this gene, which we named *frzb*, is involved in morphogenesis of the mammalian skeleton.

The discovery and identification of diffusible factors that regulate skeletal morphogenesis have dramatically improved our understanding of the molecular events governing skeletal pattern formation. Genetic studies have confirmed the importance of these differentiation factors in the formation, growth, and maintenance of the skeleton (2). Likewise, nondiffusible molecules, including components of the extracellular matrix and cell surface, are essential to patterning processes. One concept proposed for insect systems postulates that morphogenesis results from the (re)positioning of cells because of inherent characteristics such as differential adhesiveness (3). Whether analogous events occur in mammalian skeletal pattern formation is unknown.

In *Drosophila melanogaster*, the cuticle contains hairs and bristles arranged in a defined polarity, of which the pattern and orderly alignment reflect the polarity of the wing epidermis (4). Typically, these structures are aligned in parallel and point in the same direction on the body surface. Several genetic loci associated with epidermal cell polarity have been studied. One of the most thoroughly investigated is the *frizzled* (fz)[1] locus. *frizzled* encodes an integral membrane protein with seven potential transmembrane domains. This locus is required for cellular response to a tissue polarity signal as well as intercellular transmission of that signal along the proximal-distal wing axis (5, 6). Mutations of the *fz* locus result in disruption of both cell-autonomous and non-cell-autonomous functions of the *fz* gene. Strong *fz* mutations are associated with random orientation of wing hairs. Weaker mutations lead to hair and bristles oriented parallel to neighboring cells, although orientation is random with respect to the body axis (5). In addition, it was recently shown that *frizzled* regulates mirror-symmetric pattern formation in the *Drosophila* eye (7).

The homologues *frizzled-1* and *frizzled-2* (*fz-1, fz-2*) have been cloned from rat and human, and Northern analysis revealed expression in a wide variety of tissues including kidney, liver, heart, uterus, and ovary (8, 9). Six novel mammalian *frizzled* homologues have been identified recently (10). Interestingly, each of them seems to be expressed in a distinctive set of tissues during development or postnatally (10). In addition, a Frizzled-like domain has been found in one of the N-terminal noncollagenous domains of α1 (XVIII) collagen (11), although its similarity to other members of the class is comparatively low.

In an effort to identify signaling molecules involved in skeletal patterning and skeletal tissue formation, we used cartilage to isolate highly purified protein fractions with *in vivo* chondrogenic activity (1). Chondrogenic/osteogenic activity was as-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U24163 for human FRZB and U24164 for bovine Frzb.

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[1] The abbreviations used are: fz, frizzled; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; kb, kilobase pair(s); PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

FIG. 1. **Nucleotide and amino acid sequence of the full-length bovine *frzb*.** The predicted *frzb* gene product contains 325 amino acids with a putative signal peptide (*box*). *Dashed underline*, the tryptic peptide sequence used to obtain a cDNA fragment by RT-PCR. Two separate consensus polyadenylation sites are *underlined*. A termination codon "tga" is shown in the 5′-untranslated region.

sayed *in vivo* by subcutaneous implantation of ethanol-precipitated protein fractions in rats (12); implants were recovered after 10 days and analyzed histologically for alkaline phosphatase activity. Trypsin digestion of the bioactive purified fractions and isolation of the resulting peptides allowed identification of several unique peptides by amino acid sequencing. Starting from a 30-amino acid peptide, we identified a cDNA encoding a novel Frizzled-like protein. A GenBank℠ data base search revealed that this protein contained a domain of about 110 amino acids with significant homology to the N-terminal domain of *Drosophila* Frizzled and its rat homologue Fz-1. Unlike the Frizzled family of proteins, this gene does not contain seven transmembrane domains. This homology, along with its striking expression pattern in skeletal structures, suggests that this novel gene, which we named *frzb*,[2] is intimately involved in skeletal patterning.

MATERIALS AND METHODS

Protein Purification and Amino Acid Sequence Analysis—Highly purified protein preparations with *in vivo* chondrogenic activity were prepared as described (1). The *in vivo* chondrogenic activity was assayed in a subcutaneous implantation model in rats using a collagenous carrier (12, 13). Briefly, articular cartilage extracts (1.2 M guanidine HCl and 0.5% CHAPS in 50 mM Tris-HCl, pH 7.2) were enriched in activity using heparin-Sepharose affinity chromatography, molecular sieve chromatography, and concanavalin A-Sepharose chromatography. Subsequently, protein fractions from the 36–40-kDa region were obtained for bioassay by gel elution after SDS-polyacrylamide gel electrophoresis and were found to be chondrogenic. Primary sequencing data from the bioactive fractions were determined by transfer to polyvinylidene difluoride membrane for N-terminal sequencing (14) or nitrocellulose for digestion with trypsin as described previously (15, 16). Tryptic peptides were separated by reverse phase (17), and the sequence of individual peptides was determined using an Applied Biosystems model 477A sequencer with modifications (16, 18).

RT-PCR—Two degenerate oligonucleotides corresponding to the N terminus and C terminus of the 30-amino acid peptide 323 (ETVNLYTSAGCLCPPLNVNEEYLIMGYEFP) were used in RT-PCR: 323S, 5′-G-A(A/G)-AC(A/C/T)GT-(C/G)-AA(C/T)-CT(C/G/T)-TA(C/T)-AC(A/C/G/T)--3′; and 323AS, 5′-(A/G)AA-(C/T)TC-(A/G)TA-(A/C/G/T)CC-CAT-(A/C-/G/T)AT-3′. For RT-PCR, first strand cDNA synthesis was performed with 1 μg of poly(A)$^+$ or 5 μg of total RNA prepared from bovine articular chondrocytes, using random hexanucleotide primers from the cDNA Cycle Kit℠ (Invitrogen Corp.) or the antisense primer 323AS. The PCR was performed using the following conditions: 323S/323AS primer pairs were used in 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 30 s. PCR products were purified through a Probind℠ membrane (Millipore Corp.), followed by subcloning with the TA Cloning℠ System (Invitrogen Corp.). The amino acid sequence deduced from the PCR product matched the protein sequence from purified fractions. Two nondegenerate oligonucleotide probes were designed for cDNA library screening.

Isolation and Sequencing of cDNA Clones—Bovine articular cartilage total RNA was isolated as described (19). Poly(A)$^+$ RNA was isolated using the Poly(A)Tract℠ magnetic bead system (Promega). A cDNA library was constructed in UNIZAP℠XR (Stratagene) starting from bovine articular cartilage poly(A)$^+$ RNA. The nondegenerate oligonucleotides used to screen the articular cartilage cDNA library were 323.23 (5′-GCTCTGGCTGCCTGTGTCCTCCACTTAACG-3′) and 323.40 (5′-CCTCCACTTAACGTTAATGAGGAGTATCTC-3′). Plaques hybridizing to both oligonucleotides were further purified using standard plaque hybridization procedures (20). Once the bovine cDNA was isolated, PCR was used to generate a 1-kb fragment containing *Xho*I sites at both ends. This fragment, representing the bovine open reading frame, was used to screen a human placenta λgt11 cDNA library (CLONTECH). Approximately 7 × 10^5 plaques from the bovine library and 3 × 10^5 plaques from the human library were screened. Hybridizations were carried out for 24 h at 42 °C in 6 × SSC, 1 × Denhardt's solution, 0.01% yeast tRNA, and 0.05% sodium pyrophosphate. The membranes were washed to a final stringency of 3 × SSC and 0.1% SDS at 55 °C for 15 min.

All sequencing of both strands was done using the dideoxy chain termination method (21) and Sequenase version 2.0 DNA polymerase according to the manufacturer's instructions (U. S. Biochemical Corp.). The sequencing data were obtained by primer walking and from subclones of restriction fragments into pBluescript SKII (Stratagene). Compressions were resolved by performing the reactions using 7-deaza-GTP (U. S. Biochemical Corp.).

Protein Expression and Antiserum Production—The bovine open reading frame fragment was subcloned in the proper orientation into the *Xho*I site of pET-28a(+) (Novagen, Madison, WI), which contains an N-terminal stretch of 6 histidine residues to facilitate purification of expressed protein as well as a T7 tag for immunodetection. The pET-bovine open reading frame construct was used in the *Escherichia coli*-based pET System℠ to obtain bovine Frzb fusion protein. Purification of protein product from inclusion bodies with Ni-nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, Inc.) was performed using decreasing pH steps, according to the manufacturer's instructions.

Rabbits were immunized with Frzb fusion protein for 6 months, 250 μg of protein per boost, with a total of 10 injections. After immunization with fusion protein, several rabbits were immunized with a synthetic peptide of 12 amino acids (amino acids 51–63; TKMPNHLHHSTQ; Fig.

[2] *frzb* is pronounced frizbee; *frz* from *frizzled* motif and *b* from bone development.

A

```
                                                                  *
bovine  MVCGSRGGML LLPAGLLALA ALCLLRVPGA RAAACEPVRI PLCKSLPWNM   50
human   ------P----- --R------- ---------- ---------- ----------  50

bovine  TKMPNMLKHS TQANAILAIE QFEGLLGTHC SPDLLFFLCA MYAPICTIDF  100
human   ---------- ---------- ---------- ---------- ----------  100

bovine  QHEPIKPCKS VCERAQGCE PILIKYRHSW PESLACEELP VYDRGVCISP  150
human   ---------- ---------- ---------- --N------- ----------  150

bovine  EAIVTADGAD FPMDSSNGNC RGASSERCKC KPVRATQKTY FRNNYNYVIR  200
human   ---------- ---------- ---------- --I------- ----------  200

bovine  AKVKEIKTKC HDVTAVVEVK EILKASLVNI PRETVNLYTS SGCLCPPLNV  250
human   ---------- ---------- ----S----- --D------- ----------  250

bovine  NEEYLIMGYE DEERSRLLLV EGSIAEKWKD RLGKKVKRWD MKLRHLGLNT  300
human   ----I----- ---------- ---------- ---------- --------SK  300

bovine  SDSSESSDSTQ SQKPGRNSNS RQARN                            325
human   -----N----- ---S-----P -----                            325
```

B

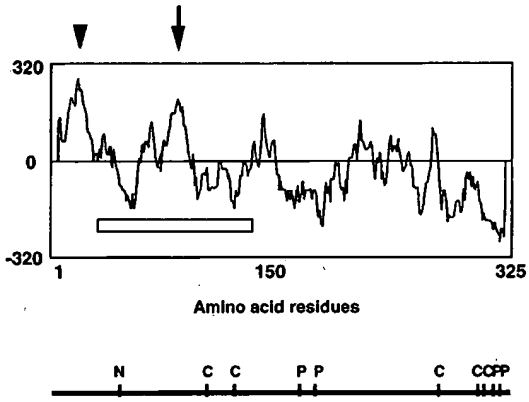

FIG. 2. *A*, deduced amino acid sequences of a cDNA clone of bovine and human *frzb*. The predicted 21-amino acid signal peptide is *boxed*. *, a potential *N*-linked glycosylation site. The putative transmembrane domain is delineated by a *bold underline*. *B*, hydropathy analysis of human FRZB from deduced amino acid sequence. The plot was generated by the GeneWorks® program using the paradigm of Kyte and Doolittle. Hydrophobic residues are in the upper part of the graph. The *arrowhead* at the N-terminal depicts the potential signal peptide. The putative transmembrane domain is indicated by a *downward arrow*. *N, C,* and *P* are *N*-glycosylation, CK2 phosphorylation, and protein kinase C phosphorylation sites, respectively. The *stippled bar* underneath the plot represents the Frizzled-like domain.

1) coupled to keyhole limpet hemocyanin through a C-terminal cysteine. The resulting antisera were screened and titered in immunoblots using the Western-Light Plus Kit® (Tropix, Inc.) according to the manufacturer's protocol. Briefly, the membranes were blocked overnight in blocking buffer (BF) consisting of 0.6% I-BLOCK® (Tropix, Inc.) in PBS and 0.1% Tween 20. The antiserum was diluted from 1:250 to 1:10,000 in BF. The membranes were washed three times for 5 min in BF after each incubation step. The membranes were then incubated with the secondary antibody at a dilution of 1:20,000 for 30 min, followed by AVDIX®(enzyme conjugate) incubation for 20 min. Blots were developed using the CSPD® chemiluminescent substrate (Tropix, Inc.) and exposed to Eastman Kodak Co. XAR-5 films for 1–10 min. Antiserum N374-PEP afforded the optimal signal:noise ratio in Western blots and was therefore selected for further studies and immunohistochemical staining.

Cell Fractionation Studies—A full-length 2.4-kb *Bam*HI-*Xho*I fragment of bovine *frzb* (Fig. 1) was cloned into the pcDNA3 expression vector (Invitrogen Corp.), and the resulting construct was named *pfrzb*. COS1 cells (1.6×10^6 initial seeding density) were transfected with 10 μg of either *pfrzb* or the control pcDNA3 vector/100-mm dish using 120 μl of LipofectAMINE® reagent (Life Technologies, Inc.). Transfection was carried out for 6 h in serum-free Opti-MEM I® (Life Technologies, Inc.). Cells were incubated at 37 °C for 72 h in serum-free Opti-MEM I® with daily medium changes. Conditioned media were then collected and concentrated 20-fold using a Centricon® 10 microconcentrator (Amicon, Inc.). Cells were scraped and resuspended in lysis buffer consisting of 10 mM Tris, 5 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. Cells were lysed using a syringe and a 25-gauge needle, and the resulting lysates were collected. Large debris, nuclei, and nonlysed cells were removed by centrifugation for 10 min at $3,000 \times g$. The supernatant was again centrifuged for 30 min at $100,000 \times g$. The pellet, which contained primarily membrane vesicles, microsomes, and other particulates, was extracted successively with 10 mM Tris-HCl, pH 8.0; 6 M urea, 10 mM Tris-HCl, pH 8.0; 1% Triton X-100 in 6 M urea, 10 mM Tris-HCl, pH 8; and finally, 1% SDS in 1% Triton X-100, 6 M urea, 10 mM Tris-HCl, pH 8. Each extraction was followed by centrifugation at $100,000 \times g$ for 30 min. The extracts were then precipitated with an equal volume of 30% trichloroacetic acid and redissolved in SDS sample buffer. Equal proportions from the cytosol, the membrane/particulate fraction, and concentrated conditioned media were loaded and separated on 4–20% gradient Tris/glycine gels (Novex), blotted to Tropifluor® polyvinylidene difluoride membrane (Tropix, Inc.) using a GENIE® electrophoretic blotter (Idea Scientific Company, Minneapolis, MN) and analyzed by immunoblotting as described above. The primary antiserum (N374-PEP) dilution was 1:1,000.

Similar cell fractionation studies were performed using primary bovine articular chondrocytes. Cells were grown to confluence in 100-mm dishes in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated for 48 h in serum-free Opti-MEM I® in the presence or absence of dextran sulfate (250 μg/ml) to possibly improve recovery of soluble protein. Conditioned media and cell layers were further processed as above.

Hybridization in Situ—Tissues from human embryos ranging from 6–13 weeks of gestation (estimated on the basis of crown-rump length and pregnancy records) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), embedded in paraffin, cut serially at 5–7 μm, and mounted on silanized slides. These tissues were obtained from legally sanctioned procedures performed at the University of Zagreb Medical School (Zagreb, Croatia). The procedure for obtaining autopsy materials was approved by the Internal Review Board of the Ethical Committee at the University of Zagreb School of Medicine and the Office of Human Subjects Research of the National Institutes of Health (Bethesda, MD). *In situ* hybridization was performed as described previously (22, 23). Briefly, after a short prehybridization, sections were incubated overnight at 50°C in 50% formamide, 10% dextran sulfate, 4 × SSC, 10 mM dithiothreitol, 1 × Denhardt's solution, 500 μg/ml freshly denatured salmon sperm DNA, and yeast tRNA with 0.2–0.4 ng/ml ^{35}S-labeled riboprobe (1×10^9 cpm/μg) in a humidified chamber. Because bovine open reading frame contained *Xho*I sites at both ends, this fragment was subcloned in both sense and antisense directions into the *Xho*I site of pBluescript SKII− vector (Stratagene), and riboprobes were made using T7 RNA polymerase according to the manufacturer's instructions (Novagen). After hybridization, the sections were washed to a final stringency of 0.1 × SSC, 65°C for 2 × 15 min. After dehydration in a graded ethanol series containing 0.3 M ammonium acetate, slides were covered with NTB-2 emulsion (Kodak) and exposed for 1–3 weeks. The slides were then stained with 0.1% toluidine blue, dehydrated, cleared with xylene, and mounted with Permount.

Immunostaining—Sections were stained using the Vectastain® *Elite* ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Before staining, the tissue sections were pretreated with chondroitinase ABC for 1 h. The sections were blocked with PBS and 10% goat serum for 30 min and subsequently incubated for 1 h with primary antiserum (N374-PEP) used at a dilution of 15 μg/ml in PBS containing 0.5% goat serum. In the controls, the primary antibody was replaced with normal preimmune rabbit serum or secondary antibody alone.

RESULTS

frzb Encodes a Protein with Regional Homology to Frizzled—To clone cDNAs corresponding to tryptic peptide 323, RT-PCR was performed using bovine articular cartilage poly(A)$^+$ RNA as a template. This yielded a 90-base pair DNA fragment encoding the proper peptide sequence (Fig. 1, *dashed underline*). Screening of a bovine articular cartilage cDNA library using two different 30-mer oligonucleotides designed from the 90-base pair fragment identified a 2.4-kb clone that hybridized to both oligonucleotides. This clone contained a

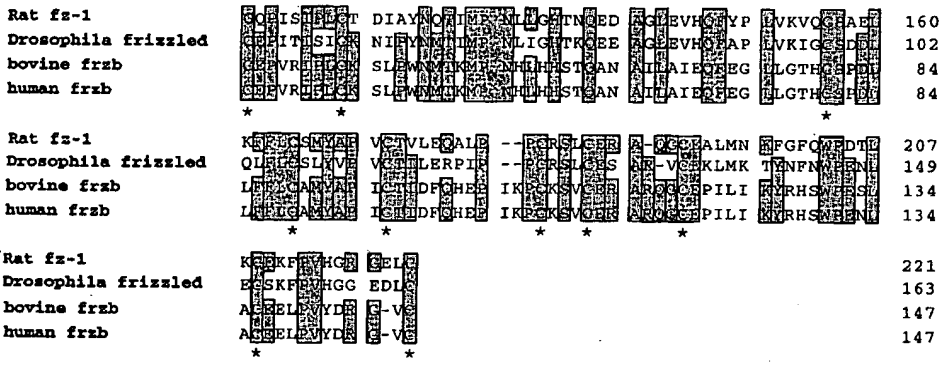

FIG. 3. **Amino acid sequence comparison of the N-terminal domain of bovine and human FRZB: homology with rat Fz-1 (8) and *Drosophila* Frizzled (6).** Identical residues are denoted by *shaded boxes*. Gaps (–) are introduced in the sequence to optimize alignment, and asterisks (*) denote sites of conserved cysteine residues. The numbers on the *right* indicate amino acid residues for each protein.

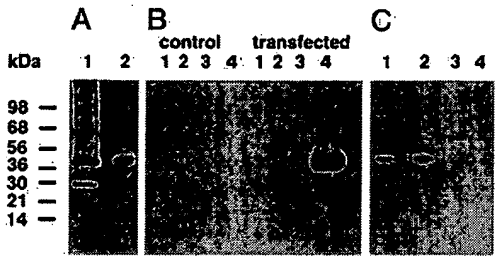

FIG. 4. **Protein expression, antibody characterization, and compartmentalization studies.** *A*, Frzb fusion protein was purified from bacterial inclusion bodies using a Ni-NTA column. Aliquots of protein fractions were run on an SDS-polyacrylamide gel according to Laemmli (29) and stained with Coomassie Blue (*lane 1*, eluate). *Left*, molecular weight standards in kDa (*lane MW*). Membrane fractions from cell lysates were prepared as described under "Materials and Methods." The immunoreactivity of this protein product was tested by immunoblotting using antiserum N374-PEP (*lane 2*). *B*, nontransfected and *frzb*-transfected COS1 cells were lysed and processed as described under "Materials and Methods." The various fractions were separated on SDS-polyacrylamide gel electrophoresis gels under reduced conditions and analyzed in immunoblots. *Lane 1*, conditioned medium; *lane 2*, cytosol; *lane 3*, 1% Triton X-100; *lane 4*, Triton X-100/urea/SDS extract. *C*, primary bovine articular chondrocytes were grown to confluence. The cultures were subsequently maintained for 48 h in serum-free conditions in the presence or absence of dextran sulfate (250 μg/ml); supernatants were collected, and cells were lysed, processed, and analyzed in immunoblots as above. *Lane 1*, membrane fraction from cell lysates with dextran sulfate; *lane 2*, without dextran sulfate; *lanes 3* and *4*, supernatants of samples shown in *lanes 1* and *2*, respectively.

single open reading frame with two separate consensus polyadenylation sites and a poly(A) tail (Fig. 1). A 1.3-kb clone contained a single polyadenylation signal, a short poly(A) tail, and a short 5′-noncoding region. Three other clones lacked the poly(A) tail but contained longer 5′-ends. Because Northern analysis using a bovine cDNA probe revealed mRNA expression in placenta (data not shown), we screened a human placenta cDNA library to isolate the human orthologue. Four clones ranging from 1.3–1.6 kb were analyzed, and all contained the same open reading frame. All clones contained a consensus translation intitiation site (24) and an in-frame termination codon situated 144 base pairs upstream of the start methionine (Fig. 1). The difference in size between the bovine and human cDNA inserts (2.4 kb *versus* 1.3 kb) is due to a longer 3′-untranslated sequence in the bovine clone (Fig. 1). Based on sequences from these overlapping cDNA clones, the predicted size of both the human and bovine protein is 325 amino acids (Fig. 2*A*).

The bovine and human amino acid sequences are 94% identical. The deduced protein sequence of both the human and bovine cDNA revealed at least four structural domains (Fig. 1; Fig. 2, *A* and *B*). An N-terminal hydrophobic stretch of 25 amino acids immediately downstream of the initiation methionine likely represents a signal peptide (25). A second hydrophobic stretch of 24 amino acids (residues 75–98), which could represent a transmembrane domain, is followed by a region with several potential serine/threonine phosphorylation sites and a serine-rich C-terminal domain (residues 301–325). Both homologues contain an *N*-linked glycosylation site at Asn-49, which is N-terminal of the putative transmembrane domain. A potential C-terminal glycosylation site in the bovine protein was not present in the human homologue.

A search of the GenBank® data base using the basic local alignment search tool network service at the National Center for Biotechnology Information (26) indicated that Frzb has significant identity (~50%) in the N-terminal region (from amino acid 35–147) to *Drosophila* Frizzled and rat Fz proteins (Fig. 3). The homologous region begins shortly after the cleavage site of the predicted signal sequence. The 10 cysteines in this region are conserved.

Frzb Is a Membrane-associated Protein—Frzb fusion protein containing six N-terminal histidines was purified from bacterial inclusion bodies using Ni-NTA affinity chromatography. The affinity-purified protein was visualized as a major band following Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis (Fig. 4*A*, *lane 1*). The identity of the fusion product was verified by immunoblotting using the T7 monoclonal antibody (data not shown). Antiserum N374-PEP (see "Materials and Methods") detected a band migrating at the same apparent molecular weight on a Western blot (Fig. 4*A*, *lane 2*).

Immunoblots of protein fractions obtained from cultured cells were performed to investigate the distribution of Frzb. Experiments were done in COS1 cells transfected with *pfrzb*. As shown in Fig. 4*B* (*lane 4*, transfected), the urea/SDS/Triton X-100 extract of the membrane pellet contained most of the frzb protein. No protein was detected in the supernatants of the transfected cells (Fig. 4*B*, transfected, *lane 1*). No protein was detected in untransfected cells (Fig. 4*B*, control).

Because the protein sequencing data were obtained from partially purified protein preparations of bovine articular cartilage extracts, immunoblots were also performed on supernatants and cell extracts of primary bovine articular chondrocyte cultures. Again, most of the protein was detected in the membrane-associated fractions (Fig. 4*C*, *lane 1*). The addition of dextran sulfate (250 μg/ml) did not change this distribution (Fig. 4*C*, *lanes 2* and *4*).

frzb Displays a Dynamic Expression Pattern in Developing Limbs—Serial sections of human embryos representing various stages of development were used for *in situ* hybridization to

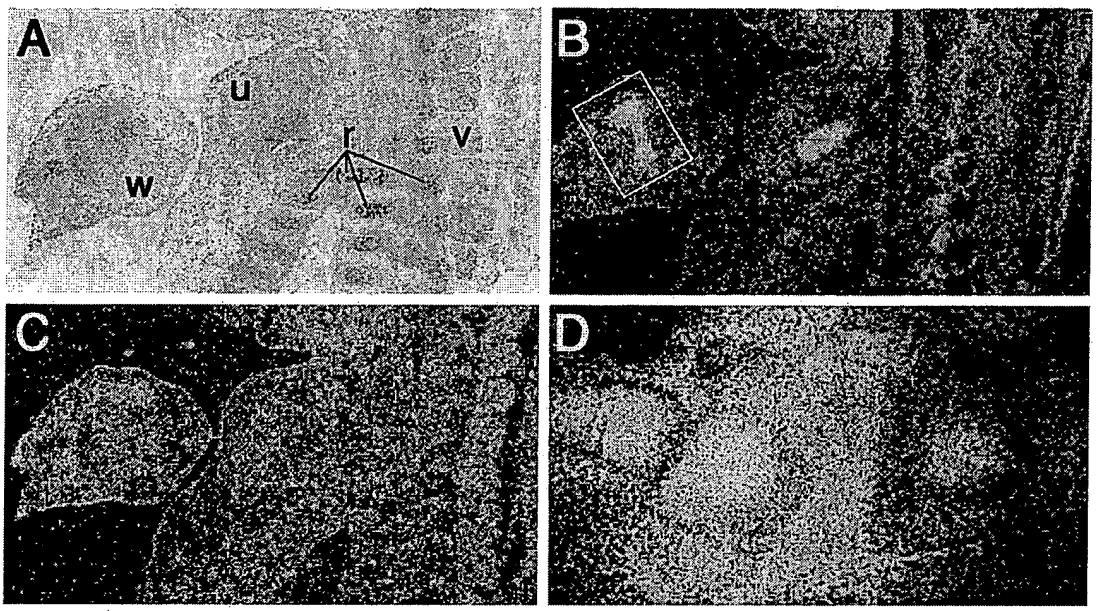

FIG. 5. ***frzb* is present in human developing limbs at 6 weeks.** *A*, toluidine blue-stained bright-field image of a 6-week-old embryo. *w*, wrist; *u*, upper arm; *r*, ribs; *v*, vertebral bodies. *B*, dark-field image of sections 24 μm away from brigh field section, hybridized with the antisense riboprobe with a strong continuous signal in the limb rudiments (*arrow*) and ends of the ribs but no signal in axial bone structures. *C*, dark-field image of a section hybridized with a *frzb* sense riboprobe. *D*, higher power image of the distal part of the limb (*boxed* in *B*), showing signal between and surrounding the cartilaginous cores.

explore the pattern of *frzb* expression during embryonic development. Between 6 and 13 weeks, no hybridization was detected in most organs, including kidney, heart, muscle, intestine, liver, brain, and lung (data not shown). In contrast, strong hybridization was seen in the developing appendicular skeleton. At 6 weeks, *frzb* transcripts were clearly visible *surrounding* the early cartilaginous rudiments of the developing limbs, as shown in the distal parts of the upper limb (Fig. 5). Hybridization is apparent between neighboring areas of cartilaginous condensation in developing long bones. Subsequently, expression appears *within* the cartilaginous cores of developing long bones. This is apparent in the proximal parts of the upper limb, which are more advanced in developmental stage than the distal parts (Fig. 5). In addition, *frzb* was detected in the cartilage anlagen of several craniofacial bones (data not shown) and the epiphysial ends of the rib cage (Fig. 5), whereas no signal was detected in the vertebral bodies at 6 weeks. At 13 weeks of gestation, *frzb* transcripts were present in early chondroblasts of the tarsal bones of the foot, the carpal bones of the hand, and the epiphysis of long bones (Fig. 6, *I* and *II*). A striking feature of the expression pattern at this developmental stage was the presence of a graded distribution, most prominent in the phalanges (Fig. 6*I*). The highest level of expression was observed at the epiphyses of long bones and the periphery of cuboidal bones (Fig. 6, *I* and *II*). The expression level then decreased with the appearance of chondrocyte hypertrophy and vascular invasion and seemed to be absent in the primary centers of ossification. Interestingly, at this stage of development, several layers of chondroblasts adjacent to the joint space did not show detectable transcripts (Fig. 6*I*). In sharp contrast to the prominent expression observed in other skeletal structures, no expression was apparent in the vertebral bodies at the stages examined (Fig. 5; data not shown).

Immunohistochemical staining confirmed the presence of protein in developing skeletal structures, appearing within the cartilaginous cores of the developing long bones (Fig. 7). The graded mRNA expression pattern detected by *in situ* hybridization, most prominent in the phalanges, was paralleled by the protein distribution (Fig. 7).

DISCUSSION

To characterize factors responsible for cartilage and bone inductive activity in articular cartilage (1), we isolated a protein fraction containing potent bone and cartilage inductive activity. Edman analysis of peptides obtained by tryptic digestion of the bioactive fraction allowed us to identify novel genes by means of RT-PCR. One of the cDNAs obtained using a PCR product encoding a 30-amino acid peptide as a hybridization probe contained an open reading frame encoding a protein of $M_r \approx 36{,}200$, which agrees well with the molecular weight of the highly purified gel-eluted protein preparations described previously (1). mRNA blots reveal a major signal at 2.4 kb, which is consistent with the size of the bovine cDNA. This clone contained an additional polyadenylation signal that could give rise to the 1.7-kb message on Northern analysis. Edman analyses of four additional peptides in the bioactive protein preparation were consistent with the sequence deduced from the cDNA. Finally, this cDNA was found by independent screening of two different cDNA libraries. These data, together with the hybridization signals obtained in genomic Southern blots (data not shown), provide strong evidence for the existence of *frzb* in diverse species. The relationship of Frzb protein to chondrogenic activity in the purified fractions of cartilage extracts is unclear. The presence of the protein in these fractions may be coincidental and thus a serendipitous finding.

The presence of a putative signal peptide suggests that Frzb enters the secretory pathway. A hydrophobic 24-amino acid stretch in the N-terminal region suggests the possibility of a transmembrane domain. The C-terminal region of the protein contains several potential serine/threonine phosphorylation sites (7 in bovine and 9 in human) and a region of high serine and threonine content (10 of the last 26 residues). Consequently, this molecule is a potential target for protein phosphorylation via serine/threonine kinases. In addition, subcellular

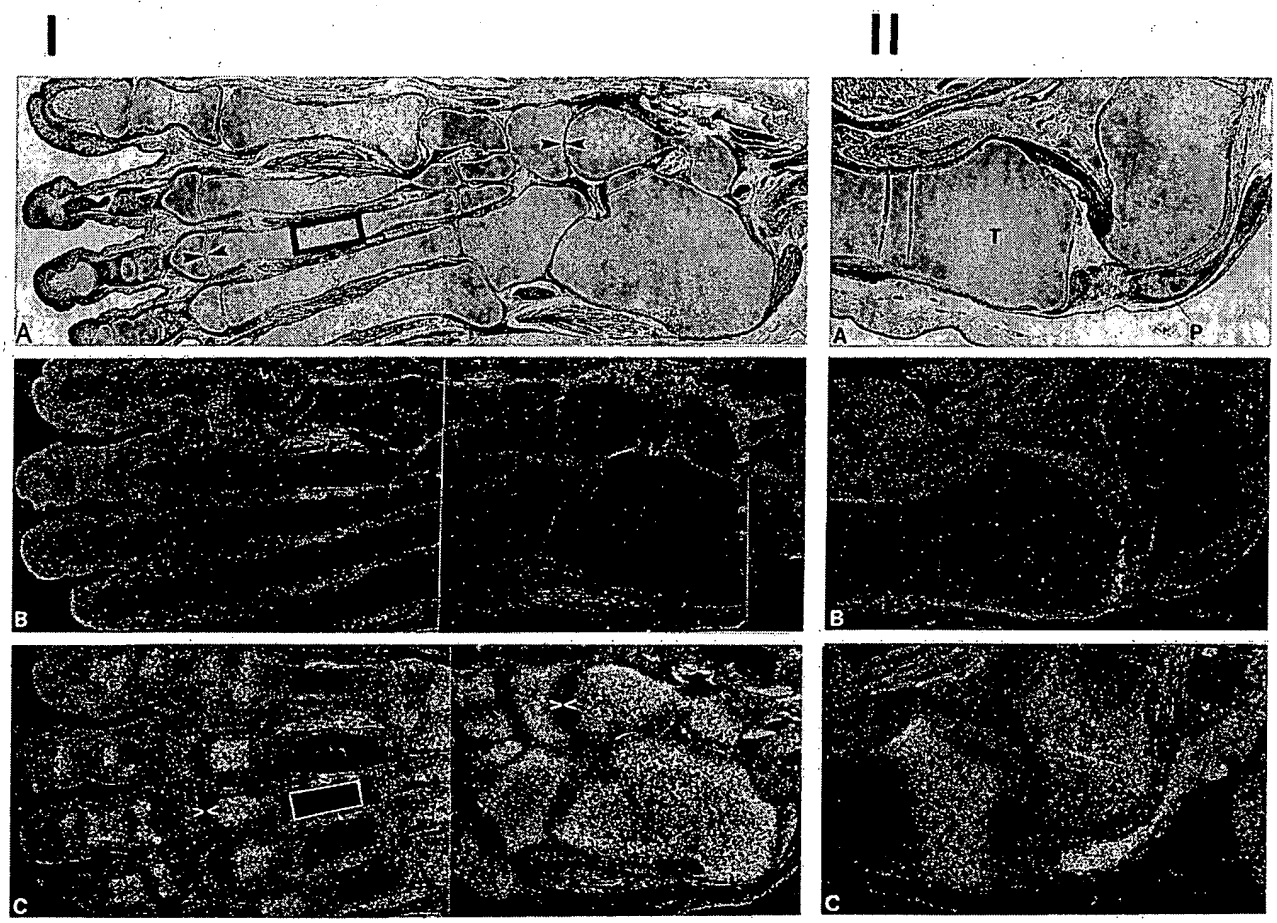

FIG. 6. ***frzb* expression pattern at 13 weeks in the appendicular skeleton.** Bright-field (*IA* and *IIA*) and dark-field images (*IB*, *IIB* and *IC*, *IIC*) of sections through a 13-week-old human embryonic foot (*I*) and knee (*II*). Hybridization was performed with both sense (*IB* and *IIB*) and antisense (*IC* and *IIC*) ^{35}S-labeled bovine riboprobe containing the open reading frame. The *boxed zones* in *IA* and *IC* indicate the site of the developing primary ossification center. *Opposing arrowheads* demarcate the joint space. *T*, tibia; *P*, patella. *Bar*, 150 μm.

fractionation experiments further suggest that Frzb is membrane-associated. The cysteines in the molecule do not seem to form intermolecular disulfide bridges, because the size of Frzb protein in highly purified cartilage preparations did not change in Western blots of SDS-polyacrylamide gel electrophoresis separations performed under nonreducing conditions after reduction and alkylation (data not shown).

A GenBank® data base search revealed striking sequence similarity of the N-terminal domain of *frzb* to the *Drosophila* polarity gene *frizzled* (6) and its mammalian homologue, the rat *fz-1* gene (8). The homologous domain of about 110 amino acids corresponds to the suggested extracellular domain of the *frizzled* gene products. Unlike the *frizzled* and *fz* gene products, Frzb lacks the seven transmembrane domains found in a large family of G-protein-coupled membrane receptors (10). Molecular structural analysis, together with our cell fractionation data, suggests that a 24-amino acid hydrophobic stretch within this domain might provide a membrane-anchoring function. Genomic sequence analysis revealed that in both *Drosophila frizzled* and *frzb*, the homologous domain is encoded by the first exon.[3] This suggests a common ancestry and function. Recently, a similar (though less highly conserved) cysteine-rich Frizzled-like domain has been found in one of the N-terminal noncollagenous domains of α1 (XVIII) collagen (11), supporting the notion that the *frizzled*-like sequence is a conserved motif.

[3] J. T. Thomas and F. P. Luyten, unpublished observations.

The basic form and pattern of the skeleton derived from lateral plate mesoderm are first recognizable when mesenchymal cells aggregate into regions of high cell density called condensations. They subsequently differentiate into cartilage and bone and continue to grow by cell proliferation, cell enlargement, and matrix deposition. Genetic studies have demonstrated that disruption of these condensations results in disturbed skeletal phenotypes (for review see Ref. 2). In humans, limb development takes place over a 4-week period from the 5th to 8th week (27). The upper limbs develop slightly in advance of the lower limbs, although by the end of the period of limb development, the two limbs are nearly synchronized. The most proximal parts of the limbs develop somewhat in advance of the more distal parts. In the developing human limb bud, *frzb* appears between and around the early cartilaginous condensations of the limb. Subsequently, transcripts are detected within the cartilaginous cores of the developing skeleton. The expression pattern in the developing long bones is graded; the highest levels appear in chondroblasts of the epiphysis and decrease toward the primary ossification center. Surprisingly, at 13 weeks of embryonic development, the expression of this gene was not observed in several layers of chondrocytes lining the joint surface of the phalanges (Fig. 6*I*), with a sharp delineation of the expression of *frzb* at the junction between the superficial (closer to the joint surface) and deeper layers of chondrocytes of the developing long bones. Immunostaining reflected this expression pattern at the protein level (Fig. 7).

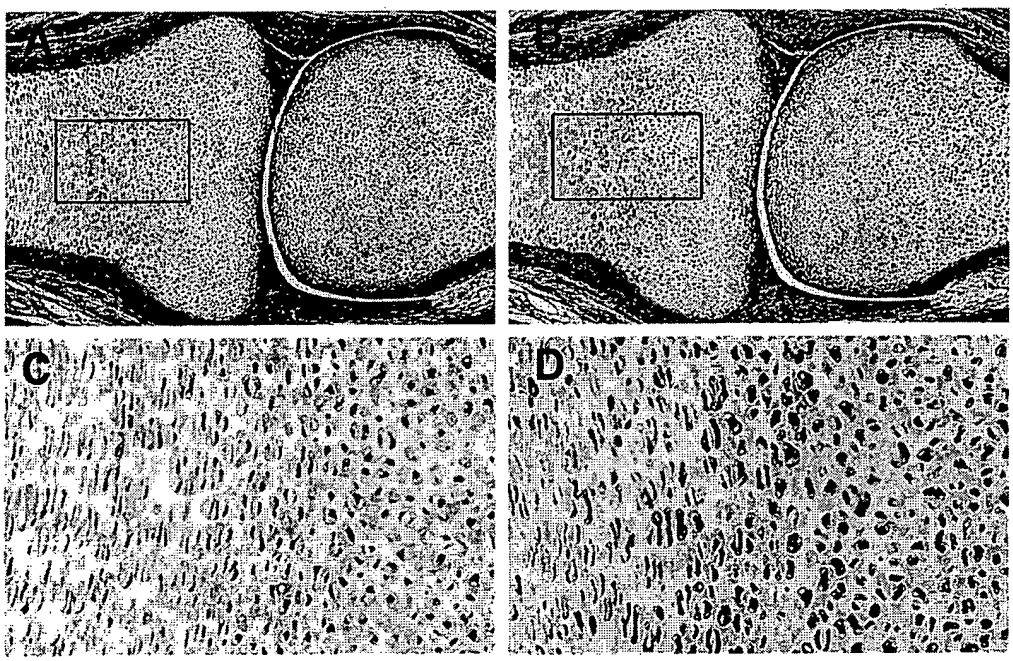

FIG. 7. **FRZB immunostaining in the developing human phalanges at 13 weeks.** *A* and *C*, preimmune antiserum staining; *B* and *D*, N374-PEP antiserum staining. Positive staining (*brown-black*) is detected in the epiphysial chondrocytes of the phalanx. No staining is seen in several layers close to the joint surface or in the chondrocytes close to the primary ossification center. Magnification: *A* and *B*, ×100; *C* and *D*, ×400.

These findings provide evidence that in early stages of embryonic development, there might already be distinct differentiation pathways for articular and epiphysial chondrocytes. No *frzb* transcripts were detected in vertebral bodies at any stage of skeletal development up to 13 weeks. These observations suggest distinct molecular pathways for specification of the axial (derived from paraxial mesoderm) as opposed to the appendicular (derived from lateral plate mesoderm) skeleton, as has been suggested previously (1, 28).

Polarity determination is thought to be a crucial step in morphogenesis and pattern formation. The homology of Frzb to Frizzled, together with a unique spatial and temporal expression pattern in developing skeletal tissues, suggests that this newly discovered protein may play a role in skeletal morphogenesis.

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Frzb, a Secreted Protein Expressed in the Spemann Organizer, Binds and Inhibits Wnt-8

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Summary

We isolated a Xenopus homolog of Frzb, a newly described protein containing an amino-terminal Frizzled motif. It dorsalized Xenopus embryos and was expressed in the Spemann organizer during early gastrulation. Unlike Frizzled proteins, endogenous Frzb was soluble. Frzb was secretable and could act across cell boundaries. In several functional assays, Frzb antagonized Xwnt-8, a proposed ventralizing factor with an expression pattern complementary to that of Frzb. Furthermore, Frzb blocked induction of MyoD, an action reported recently for a dominant-negative Xwnt-8. Frzb coimmunoprecipitated with Wnt proteins, providing direct biochemical evidence for Frzb-Wnt interactions. These observations implicate Frzb in axial patterning and support the concept that Frzb binds and inactivates Xwnt-8 during gastrulation, preventing inappropriate ventral signaling in developing dorsal tissues.

Introduction

Classical transplantation experiments established that anatomically discrete regions of early vertebrate embryos control patterning of both the developing body axis (Spemann and Mangold, 1924) and limb (Harrison, 1918; Saunders and Gasseling, 1968; Tickle et al., 1975) and suggested that diffusible factors could mediate these effects. Decades later, Urist (1965) reported induction of ectopic skeletal tissue by a protein fraction from demineralized bone and explicitly compared this phenomenon to that of embryonic induction. In the past several years, the number of secreted factors implicated in both limb and axial patterning has increased steadily (Sive, 1993; Dawid, 1994a, 1994b; Kessler and Melton, 1994; Slack, 1994; Hogan, 1996).

Some of these, including noggin, follistatin, chordin, and the nodal-related genes, are expressed in the organizer, the region implicated by Spemann in specification of the dorsal axis (Spemann and Mangold, 1924). In contrast, BMP-4 and Xwnt-8 are expressed in presumptive ventral mesoderm and endoderm early in gastrulation and are thought to act as positive ventral inducers (Christian et al., 1991; Christian and Moon, 1993; De Robertis and Sasai, 1996; Hogan, 1996; Hemmati-Brivanlou and Melton, 1997). Noggin (Zimmerman et al., 1996), chordin (Piccolo et al., 1996), and follistatin (Hemmati-Brivanlou et al., 1994) are thought to produce their dorsalizing effects by binding to BMP-4 or a related TGF-β class signal and inactivating it. No secreted factor with Wnt binding activity has been identified to date.

Another puzzle presented by the Wnt proteins—of great interest in view of their participation in a wide variety of developmental and neoplastic processes (Nusse and Varmus, 1992; Moon et al., 1993; Moon, 1993; Parr and McMahon, 1994)—has been the identity of their receptors. Recently, Frizzled class proteins were proposed as receptors for the Wnt growth factors (Wang et al., 1996). This concept was supported further by the observation that Wingless protein (Wg), the Drosophila prototype of the Wnt family, binds to cells transfected with the Drosophila *frizzled2* gene (*Dfz2*). Moreover, addition of Wg to cells transfected with *Dfz2* causes increased accumulation of Armadillo, a Drosophila homolog of β-catenin; this is an expected consequence of Wg signaling (Bhanot et al., 1996). In Xenopus embryos, overexpression of rat *frizzled-1* (*Rfz-1*) resulted in recruitment of Xwnt-8 and Xenopus Dishevelled, a component of the Wnt signaling pathway, to the plasma membrane (Yang-Snyder et al., 1996), providing additional evidence for interactions between Frizzled and Wnt proteins.

Recently, we identified a novel protein, Frzb, in a highly purified fraction from bovine articular cartilage (Hoang et al., 1996). Though this fraction contained potent chondrogenic/osteogenic activity, extensive peptide sequence analysis failed to reveal known classes of proteins associated with specification of skeletal tissues (M. M., Jr., and F. P. L., unpublished data). However, the amino terminal region of Frzb is quite similar to the putative receptor binding domain of the *frizzled* product (Vinson and Adler, 1987), which has been implicated in polarity determination in Drosophila. This finding, together with its dynamic expression pattern in developing limbs, suggested that Frzb may contribute to pattern formation in vertebrate limb development. The parallels between axial and limb patterning noted earlier prompted us to exploit the experimental advantages afforded by assays of axial patterning in Xenopus embryos to explore the biological activities of Frzb. Since the amino-terminal region of Frzb is highly similar in primary structure to the putative ligand binding domains of the Frizzled proteins, we also investigated the possibility of both protein–protein and functional interactions between Frzb and members of the Wnt family.

In the present work, we demonstrate that Frzb shows dorsalizing activity in Xenopus embryos. It is expressed in the Spemann organizer, a region critical to patterning of the amphibian embryo. This pattern is complementary to that of Xwnt-8, a secreted protein thought to ventralize mesoderm during gastrulation (Christian and Moon, 1993). Naturally occurring Frzb protein can be identified biochemically during gastrulation. It is soluble and secretable and can act across cell boundaries. Of

particular interest, Frzb binds to Xwnt-8 directly and blocks its activity in several functional assays in vivo. Furthermore, Frzb blocks induction of MyoD, an action reported recently for a dominant-negative Xwnt-8 (Hoppler et al., 1996). These lines of evidence lead us to propose that Frzb can act as a functional inhibitor of Wnt signaling through direct extracellular binding. It could thus play an important role in dorsoventral patterning of the mesoderm, as well as other processes modulated by Wnt signaling.

Results

Frzb Can Dorsalize Embryos but Not Animal Cap Explants

As an initial test of the possibility that Frzb may play a role in patterning of the vertebrate embryo, we evaluated the effects of overexpression and ectopic expression in developing embryos. Injection of bovine *frzb* (*Bfrzb*) mRNA into single ventral blastomeres at the four cell stage generated duplicated partial posterior dorsal axes reproducibly (Figure 1C). Muscle and neural tissues were apparent in frontal sections taken from these embryos (Figure 1D), but notochord was absent. The frequency of axis duplication was approximately 15% (24/159; four independent experiments) with bovine Frzb; it was somewhat less with the Xenopus gene. This difference may be due to the presence of a consensus translation initiation site (Kozak, 1991) in the bovine, but not the amphibian sequence. The phenotypes were identical in either case. When *frzb* mRNA was injected into UV-irradiated embryos, dorsal axes were partially rescued in approximately 56% (37/66; three independent experiments; Figure 1G). The rescued axes contained muscle and neural tube, but no notochord (Figure 1H). Nevertheless, overexpression of *frzb* in animal cap explants did not induce markers for mesoderm (*Brachyury* [*Xbra*]), neural tube (*NCAM*), or somites (*muscle actin*) (not shown).

Xenopus *frzb* Is a Zygotic Transcript Expressed in the Dorsal Blastopore Lip

The results of our initial functional assays prompted us to isolate a Xenopus homolog of *frzb* (*Xfrzb*). We isolated several clones with similar sequences from a Xenopus neurula cDNA library by conventional low-stringency hybridization. The clone selected for analysis was designated *Xfrzb-1b*. A closely related gene with similar characteristics, *Xfrzb-1a*, which probably corresponds to an alternative allele, has also been identified (Leyns et al., 1997 [this issue of *Cell*]). For simplicity, we refer to *Xfrzb-1b* as *Xfrzb* in the remainder of this report. The amino-terminal domain of Xfrzb was 92% identical in amino acid sequence to the mammalian orthologs; the overall identity was 72%. The regions of lowest similarity correspond to the putative signal sequences and the exon–intron boundaries of mammalian *frzb* genes (J. Terrig Thomas and F. P. L., unpublished data), which characteristically are not conserved across species. Xfrzb shares several features common to the mammalian genes described earlier (Hoang et al., 1996), including a consensus site for asparagine-linked glycosylation, a conserved cysteine-rich domain characteristic of

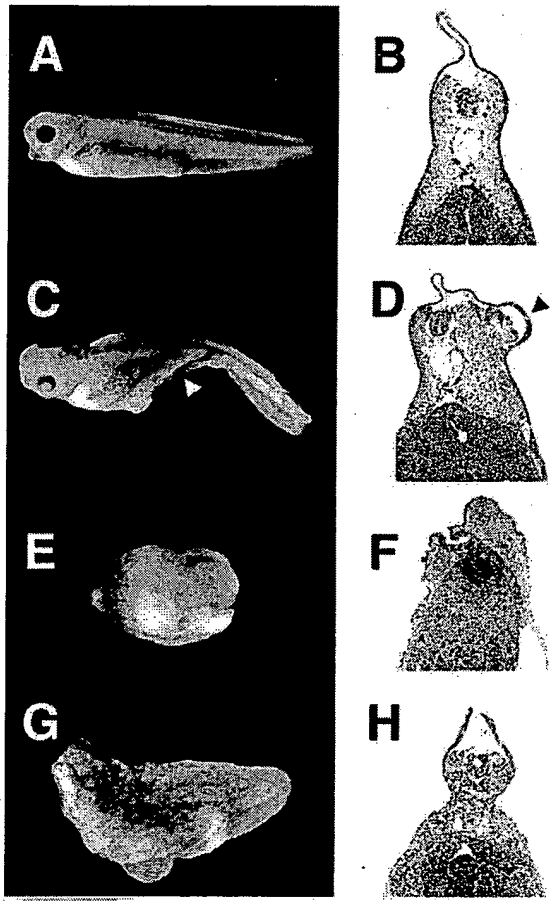

Figure 1. Partial Axis Duplication and Rescue by *frzb*

Control embryos are shown in (A) and (B). Of the embryos injected with 1 ng of *Bfrzb* mRNA into single ventral blastomeres at the 4–8 cell stage, 15% developed partial secondary axes (C and D), as indicated by arrows. Nearly all of the UV-irradiated embryos (E and F) were ventralized completely. 56% of the UV-irradiated embryos injected with 1 ng of *frzb* mRNA showed partial rescue of a dorsal axis as shown (G and H); the other embryos remained completely ventralized. (B), (D), (F), and (H) are frontal sections. The partial secondary axis in (C) and (D) and the rescued axis in (G) and (H) contain muscle and neural tissue, but no notochord. Nevertheless, *frzb* overexpression in animal cap explants did not induce mesoderm or dorsal markers (see text for details).

Frizzled proteins, and a carboxyl-terminal motif (amino acids 244–293) that appears to be homologous to the netrin-specific carboxyl-terminal domain of C. elegans unc-6 (Wadsworth et al., 1996).

Xfrzb expression first became apparent in the late blastula (stage 9) by hybridization in situ (Figure 2A, 9V). In early gastrulas (stage 10), mRNA expression was most apparent in the Spemann organizer. In later gastrulas (stage 10.5–11), there was expression in the blastopore lip that extended beyond the organizer as the blastopore lip progressed ventrally. At about stage 11, *Xfrzb* expression appeared in the dorsal midline. Examination of cleared embryos and corresponding histological sections revealed that this expression was in the involuted

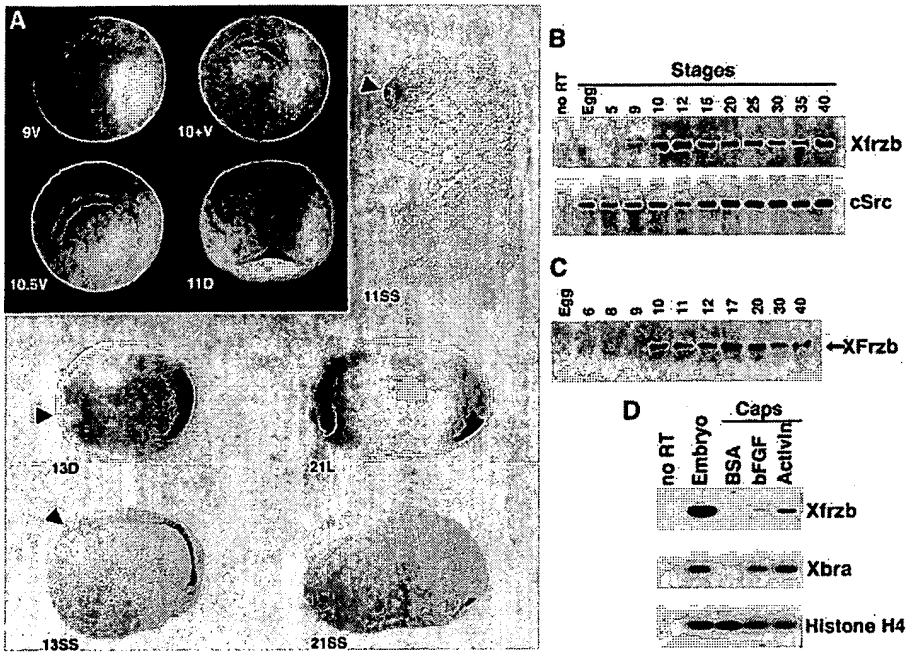

Figure 2. Expression of Frzb during Xenopus Development

(A) Localization of *Xfrzb* mRNA during early development by whole-mount hybridization in situ. Numbers indicate embryonic stages. (V), vegetal view; (D), dorsal view; (SS), sagittal section; (L), lateral view. All embryos except the dorsal views (11D and 13D) are shown with dorsal on top; the stage 13 and 21 embryos are presented with anterior to the right. High levels of *Xfrzb* expression are identified by dark purple-blue staining. In the late blastula (stage 9) and early gastrula (stage 10), expression is most prominent in the region of the Spemann organizer. The red arrowhead (stage 11SS) indicates the dorsal lip of the blastopore; this section demonstrates hybridization in the involuted dorsal mesoderm. Black arrowheads identify the yolk plug; the cleared early neurula (stage 13D) and corresponding section (13SS) both show prominent staining in anterior mesoderm. By the neural tube stage (21L, 21SS), *Frzb* is expressed primarily in anterior and posterior mesoderm.

(B) Expression levels of *Xfrzb* mRNA. RT–PCR with total RNA isolated from the indicated stages was performed for *Xfrzb*; *cSrc* was used to confirm similar amounts of input cDNA between samples.

(C) Immunoblot analysis of endogenous Frzb protein. Each lane represents 0.5 embryo. Specific staining is indicated by the arrow. These analyses were performed twice with similar results, and they confirm the presence of endogenous Frzb protein in developing embryos.

(D) Induction of *Xfrzb* by activin but not bFGF. Animal caps were explanted at stage 8 and cultured until sibling embryos reached stage 11. RT–PCR analysis of explants incubated with BSA, bFGF (20 ng/mL), or activin (5 U/mL) indicated that *Xfrzb* behaves as a dorsal marker. cDNA from whole embryos (Embryo) was used as a positive control template. *Brachyury* (*Xbra*), a general mesodermal marker known to be induced by both growth factors, is used as a positive control for mesoderm induction. *Histone H4* was used to confirm similar amounts of input cDNA between samples. For both RT–PCR assays, reactions were done with template from which reverse transcriptase was omitted (no RT) to control contamination.

mesoderm (Figure 2A, 11SS). Near the onset of neurulation, posterior expression was markedly reduced, and expression in the prechordal plate became apparent (Figure 2A; 13D, 13SS). The field of expression was then restricted progressively, stabilizing in the putative pituitary, and posteriorly in the vicinity of the proctodeum (Figure 2A, 21L; 21SS). These results are consistent with RT–PCR analysis (Figure 2B).

Endogenous Xenopus Frzb protein could be detected in early gastrulas (stage 10, Figure 2C) and in all subsequent stages analyzed by immunoblot analysis. *Xfrzb* expression was unaffected by bFGF, enhanced by activin (Figure 2D) or lithium (not shown), and suppressed by UV irradiation (not shown), as has been described for other genes expressed in the organizer (Kao and Elinson, 1988; Slack, 1994).

Frzb Blocks Wnt-8 Signaling In Vivo

The complementary relationship between the expression patterns of *Xwnt-8* and *Xfrzb* (Christian et al., 1991; Smith and Harland, 1991; Christian and Moon, 1993; Figure 7) resembled those observed for *BMP-4* and its functional antagonists, *chordin* (Piccolo et al., 1996) and *noggin* (Zimmerman et al., 1996). We therefore evaluated the possibility of an analogous functional interaction between Frzb and Xwnt-8. When *Xwnt-8* mRNA is injected during early embryogenesis, secondary dorsal axes with complete head structures are induced reliably (Smith and Harland, 1991; Sokol et al., 1991). This phenomenon can thus be used as an in vivo assay for Xwnt-8 activity. Accordingly, we tested whether coinjection of *Xfrzb* mRNA could influence formation of secondary axes (Figures 3A–3C). When *prolactin* mRNA was coinjected with *Xwnt-8* message, 71% of the embryos (in the experiment shown, 27/38) developed secondary axes. In contrast, when the *prolactin* mRNA was replaced by an identical amount of *frzb* message, axis duplications were suppressed (0/32 for *Xfrzb*; 1/36 for *Bfrzb*). Uninjected embryos did not display axial abnormalities (0/59).

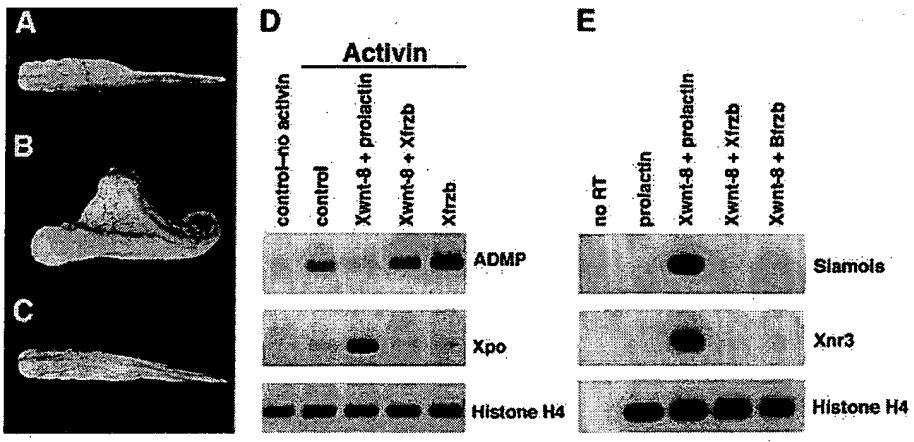

Figure 3. Frzb Blocks Wnt-8 Signaling In Vivo

(A–C) *Xfrzb* blocks induction of secondary axes by *Xwnt-8*. Embryos injected with 25 pg *preprolactin* mRNA (A), 5 pg *Xwnt-8*^{myc} mRNA, and 25 pg *preprolactin* mRNA (B), or 5 pg *Xwnt-8*^{myc} mRNA and 25 pg *Xfrzb* mRNA (C). Dorsal views are shown, with anterior to the left. All injections were into a single ventral vegetal blastomere at the eight cell stage. This experiment was done twice with *Xfrzb* and twice with *Bfrzb* with identical results. In the experiment shown, the group corresponding to panel (A) showed 0/56 secondary axes; to panel (B), 27/38 secondary axes; and to panel (C), 0/32 secondary axes.

(D) Frzb blocks ventralization of animal cap explants by CSKA-*Xwnt-8* plasmid. Embryos were injected with the indicated combinations of *prolactin* or *Xfrzb* mRNA (1 ng) and CSKA-*Xwnt-8* expression plasmid (100 pg) into both blastomeres at the two cell stage. Caps were explanted at stage 8 and incubated in the absence and presence of activin (5 U/mL) until stage 11. The dorsal marker *ADMP* but not the ventral marker *Xpo* was induced in animal caps cultured in the presence of activin. Post-MBT overexpression of Xwnt-8 in cap explants reversed this pattern; when *Xfrzb* was coexpressed with Xwnt-8, these effects were blocked.

(E) Frzb prevents induction of Xwnt-8 response genes. Animal cap assays were as in (D), but no activin was used. Coinjection of either Xenopus or bovine *frzb* mRNA (100 pg) blocked induction of *Siamois* and *Xnr3* by *Xwnt-8* (10 pg mRNA).

Xwnt-8 is felt to exert its primary effects on pattern formation during gastrulation (Christian and Moon, 1993). It was therefore appropriate to determine whether Frzb could antagonize the action of Xwnt-8 expressed following the midblastula transition (MBT). An *Xwnt-8* expression plasmid under the control of the cytoskeletal actin (CSKA) promotor has been shown to induce the ventrolateral marker *Xpo* (Sato and Sargent, 1991) and suppress induction of the dorsal marker *goosecoid* in activin-treated animal cap explants (Hoppler et al., 1996); this effect was blocked completely in caps overexpressing a dominant-negative Xwnt-8. Our results confirmed that *Xpo* expression could be increased by *Xwnt-8* plasmid in activin-treated animal cap explants and that *Xfrzb* could block this effect (Figure 3D). *ADMP* is a Spemann organizer–specific marker that is induced by activin in animal cap explants (Moos et al., 1995). Induction of *ADMP* by activin was suppressed in explants injected with *Xwnt-8* plasmid; this suppression was rescued by Xfrzb (Figure 3D). *frzb* overexpression did not affect the expression level of *Xwnt-8* (not shown). These observations are consisent with the interpretation that Frzb exerts its dorsalizing effects by inhibiting the action of Xwnt-8. In a related experiment, we injected the CSKA-*Xwnt-8* plasmid into dorsal blastomeres with or without *Xfrzb* mRNA. In this assay, CSKA-*Xwnt-8* plasmid produced head defects (64/80 embryos, three independent experiments), as described previously (Christian and Moon, 1993). If *Xfrzb* mRNA was coinjected with the CSKA-*Xwnt-8* plasmid, these defects were not observed (0/81 embryos).

Induction of *Siamois* (Lemaire et al., 1995) and *Xnr3* (Smith et al., 1995) in animal cap explants injected with *Xwnt-8* mRNA has been used to assay Xwnt-8 signaling (Carnac et al., 1996; Yang-Snyder et al., 1996). We therefore tested the ability of Frzb to block Xwnt-8-mediated induction of these response genes. Either *Xfrzb* or *Bfrzb* blocked the induction of both *Siamois* and *Xnr3* by Xwnt-8 (Figure 3E).

Frzb Is a Soluble, Secreted Protein That Can Act across Cell Boundaries

Since initial experiments with mammalian Frzb expressed in cell culture suggested that it may be membrane-associated (Hoang et al., 1996), we evaluated the subcellular distribution of the protein expressed in vivo. Endogenous Xfrzb protein was found in 105,000 × g supernatants isolated from Xenopus embryos (Figure 4A, lane 2) but could not be detected in the pellets (Figure 4A, lane 3). Further, Frzb protein was secreted by oocytes injected with *frzb* mRNA (Figure 4A, lane 5). The apparent molecular weight of 33 kDa is consistent with removal of the signal sequence at the cleavage site predicted (Nielsen et al., 1997) between amino acids 28 and 29; proteolytic processing likely accounts for the difference in molecular weight between secreted Frzb (Figure 4A, lane 5) and Frzb contained in oocyte lysates (Figure 4A, lane 6). Further experiments identified mammalian cell lines and culture conditions in which Frzb was secreted (not shown).

To demonstrate that Frzb can act across cell boundaries, we adapted an experimental design used to study the dominant-negative Xwnt-8 (Hoppler et al., 1996). As shown in Figure 4B, *Xfrzb* reduced the percentage of

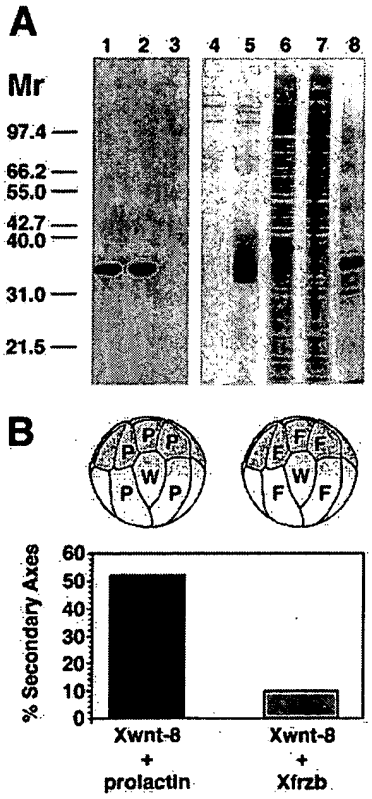

Figure 4. Frzb Is a Soluble, Secreted Protein That Can Act across Cell Boundaries

(A) Immunoblot analysis of normal stage 30 embryos (lanes 1–3) and metabolic labeling pattern of oocytes injected with *Xfrzb* mRNA (lanes 4–8). Lane 1, 20,000 × g supernatant; lane 2, 105,000 × g supernatant; lane 3, 105,000 × g pellet. Frzb was recovered in the soluble fraction. Lane 4, culture supernatant from uninjected oocytes; lane 5, supernatant from oocytes injected with *Xfrzb* mRNA; lane 6, lysate from oocytes injected with *Xfrzb* mRNA; lane 7, lysate from uninjected oocytes; lane 8, bovine Frzb expressed in E. coli. The band in lane 5 corresponds to secreted protein that has undergone proteolytic processing. The somewhat larger band in lane 6 corresponds to unprocessed protein.

(B) Frzb can block Xwnt-8 signaling across cell boundaries. Ventral blastomeres were injected with either *prolactin* (P) or *Xfrzb* (F) mRNA (50–100 pg per blastomere), as shown at the early 16 cell stage. At the late 16 cell stage, single blastomeres surrounded by those injected previously were injected with *Xwnt-8* (W) mRNA (10 pg). They were then scored for secondary axes. This experiment was performed three times with similar results; the data were pooled for the graph presented.

secondary axes induced by *Xwnt-8* from 52% (46/88) to 10% (5/49) when the two mRNAs were injected into different cells.

Frzb and Wnt Proteins Interact Directly

Direct interaction between Frzb and Wnt proteins was demonstrated in two systems: rabbit reticulocyte lysate containing canine microsomal membranes and transfected COS7 cells. *Xwnt-8^{myc}*, *Bfrzb*, *Xfrzb*, and the *β-lactamase* control mRNA were all translated and processed in vitro (Figure 5A), either alone or in the combinations indicated. As expected, the anti-myc antibody precipitated Xwnt-8^{myc} but not β-lactamase, Xfrzb, or Bfrzb (Bfrzb is not shown). Conversely, the 374-PEP antiserum, which recognized both mammalian and amphibian Frzb in immunoblots, precipitated both Xfrzb and Bfrzb (the latter is not shown), but neither Xwnt-8^{myc} nor β-lactamase. However, when Xwnt-8^{myc} and Frzb were cotranslated, both proteins were precipitated by either the myc-specific 9E10 monoclonal antibody or the 374-PEP antiserum (Figure 5B; identical results obtained with Bfrzb are not shown). Neither reagent precipitated β-lactamase cotranslated with Frzb or Xwnt-8^{myc}.

These results were further supported by experiments in which COS7 cells were cotransfected with expression plasmids encoding Bfrzb and an HA-tagged murine Wnt-1, which belongs to the same functional class as Xwnt-8 (Figure 5C) (Nusse and Varmus, 1992). Cell lysates were immunoprecipitated with an anti-HA antibody, immunoblotted, and probed with the Frzb-specific 374-PEP serum. Frzb protein was detected only in lysates from cells transfected with both *frzb* and *Wnt-1* cDNAs.

Frzb Blocks MyoD Expression

Xwnt-8 was recently implicated in somite development (Hoppler et al., 1996) through the use of a carboxyl-terminal deletion construct that acted in a dominant negative fashion. Since our data suggested that Frzb could also act as a Wnt inhibitor, we evaluated its effects on somite formation and *MyoD* expression, both of which are suppressed by the dominant-negative Xwnt-8. When *Xfrzb* mRNA was injected radially into all blastomeres at the four cell stage, trunk development was grossly abnormal (Figure 6B), resembling that seen in embryos overexpressing the dominant-negative Xwnt-8. Furthermore, *Xfrzb* blocked *MyoD* expression both in gastrulating embryos (Figure 6D) and in activin-treated animal cap explants (Figure 6E).

Discussion

Frzb May Act in Both Axial and Limb Patterning

Mammalian Frzb was first identified in a highly purified protein fraction isolated from bovine articular cartilage. This fraction contained potent osteoinductive activity (Hoang et al., 1996). Its similarity to Drosophila *frizzled*, a gene implicated in polarity determination, and its graded expression pattern in developing mammalian limbs suggested a potential role for *frzb* in embryonic pattern formation. We therefore evaluated the activity of bovine *frzb* in several assays of axial patterning. The results presented in Figure 1 indicated that mammalian *frzb* could indeed influence axial patterning in Xenopus embryos. When overexpressed ventrally, *Bfrzb* was able to induce secondary axes containing dorsal structures in normal embryos and rescue partial dorsal axes in UV-irradiated embryos, which are incapable of axis formation. These findings prompted us to identify and characterize an orthologous gene in Xenopus and examine its expression pattern and biological activities in greater detail.

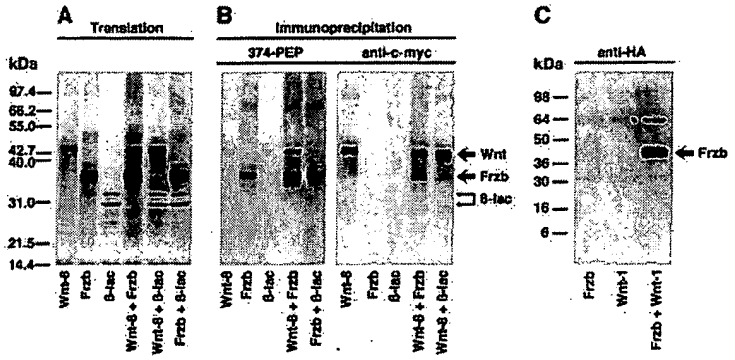

Figure 5. Frzb Binds to Wnt Proteins Directly

(A) In vitro translation of *Xwnt-8*^{myc}, *Xfrzb*, β-*lactamase*, or the indicated combinations of these mRNAs. Each of these proteins contains a signal sequence; the observed patterns are consistent with a mixture of processed and unprocessed translation products.

(B) Immunoprecipitation of in vitro translation products. The antiserum 374-PEP was used to immunoprecipitate Frzb; a commercial monoclonal was used to precipitate myc-tagged Xwnt-8. β-lactamase was used as a control for nonspecific interactions. When Frzb and Xwnt-8 were cotranslated, either antibody precipitated both proteins. These analyses were done at least twice with both Xenopus and bovine Frzb.

(C) Coimmunoprecipitation of Frzb and Wnt-1 from COS7 cells. Cells were transfected with expression plasmids encoding Frzb, an HA-tagged Wnt-1, or both together. They were then lysed, immunoprecipitated with an anti-HA antibody, and immunoblotted with the 374-PEP antiserum to detect Frzb. The specific band is indicated by the arrow. Frzb was detected in the immunoprecipitate only if cotransfected with Wnt-1. This experiment was performed at least four times with identical results.

The Expression Pattern of *Xfrzb* Is Consistent with a Role in Axial Patterning

Xfrzb is expressed in the Spemann organizer, a region of the developing amphibian embryo associated with a variety of signals that act in concert to specify dorsoventral patterning. By stage 10, endogenous Xfrzb protein could be detected by immunoblot analysis. Later in gastrulation, *Xfrzb* is expressed in the involuted mesoderm, which is thought to convey to the overlying neuroectoderm signals that participate in specification of the nervous system. Expression then becomes progressively restricted to prechordal mesoderm and finally to the putative pituitary. A region of expression remains near the proctodeum, which may be associated with residual organizer activity (Gont et al., 1993). Thus, *Xfrzb* is expressed at the appropriate time and place to participate in specification of the body axis.

Frzb Can Be Secreted

The finding that Frzb was secreted both by mRNA-injected oocytes (Figure 4A) and transfected COS7 cells suggested that it could act extracellularly. In earlier experiments, solubilization of mammalian Frzb required guanidine or sodium dodecyl sulfate (Hoang et al., 1996). Immunoblot analyses suggested that the protein examined in these experiments contained signal peptide. In contrast, immunoblot analysis of supernatants from normal embryos indicated a molecular weight consistent with removal of the signal peptide. Release of soluble protein may thus be dependent on proteolytic processing, which may vary with the cell or tissue type. When *Xfrzb* and *Xwnt-8* were overexpressed in different cells (Figure 4B), Xwnt-8 signaling was inhibited. This finding indicates that effects of Frzb on Xwnt-8 occur following secretion.

The Effects of Frzb Overexpression Depend on Cellular Context

Overexpression of Frzb can induce partial dorsal axes containing muscle (Figure 1C) or suppress *MyoD* expression (Figure 6), effects which may appear incompatible. These observations can be reconciled by consideration of the cellular context in which overexpression of Frzb occurs. Ectopic gene expression may generate a secondary axis directly by an inductive effect or indirectly by inhibition of a ventralizing signal. Frzb blocks the actions of Xwnt-8 but does not induce mesoderm, muscle, or neural tissue when overexpressed in animal cap explants, which do not express Xwnt-8. The dorsalizing actions of Frzb are thus likely to be indirect, resulting from inhibition of the ventralizing effects of Xwnt-8.

Local overexpression of a molecule acting in such an indirect manner will produce effects different from generalized overexpression. Injection of *frzb* mRNA into a single blastomere within the expression domain of *Xwnt-8* would be expected to block its ventralizing activity locally. Generation of a partial dorsal axis by *frzb* (Figure 1C) is consistent with this prediction. On the other hand, generalized overexpression should block all actions of Xwnt-8 throughout the embryo, including both its ventralizing activity and its effects on somite formation.

Recently, a dominant-negative Xwnt-8 was reported to suppress development of the trunk and somites (Hoppler et al., 1996). When Frzb was overexpressed using an identical protocol (all blastomeres at the four cell stage), the same phenotype was produced (Figure 6B). Thus, the induction of muscle tissue by local overexpression of Frzb in one type of experiment and suppression of somite development by generalized overexpression in another are compatible findings consistent with the interpretation that Frzb acts through inhibition of Xwnt-8 signaling.

Frzb Is a Functional Antagonist of Xwnt-8 In Vivo

The similarity in primary structure between Frzb and the putative ligand binding domain of the Frizzled proteins and the recent identification of these proteins as potential receptors for the Wnt family of growth factors (Bhanot et al., 1996; Wang et al., 1996; Yang-Snyder et al., 1996) provided strong impetus to examine the possibility of functional interactions between Frzb and Wnts. When overexpressed ventrally prior to the midblastula transition (MBT), Xwnt-8 is a powerful inducer of secondary

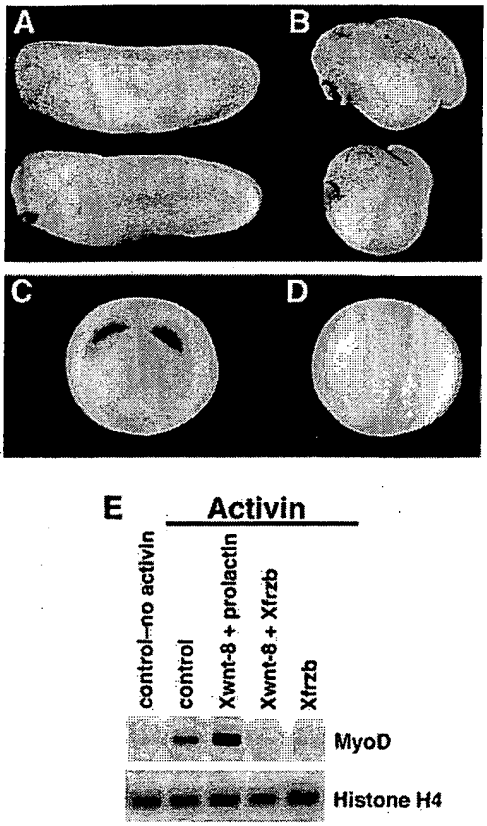

Figure 6. Frzb Blocks *MyoD* Expression

Embryos were injected at the four cell stage with 500 pg *prolactin* (A and C) or *Xfrzb* (B and D) mRNA into the marginal zone of each blastomere (instead of a single ventral blastomere as in Figure 1). Though radial injection of *Xfrzb* mRNA produced severe shortening of the trunk, anterior structures (cement gland, eyes) were present. *MyoD* expression in control (prolactin-injected) stage 11 embryos is shown in (C). Ubiquitous overexpression of *Xfrzb* completely blocked expression of *MyoD* (D). (E) shows RT–PCR analysis of animal cap explants as in Figure 3D. *Xfrzb* blocked induction of *MyoD* by activin.

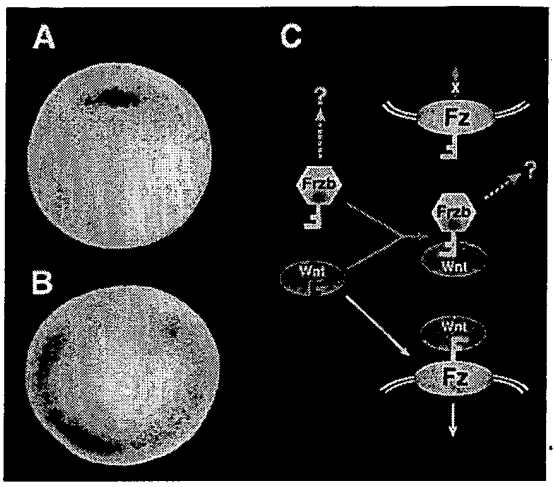

Figure 7. Proposed Interaction of Frzb and Wnt-8 in Dorsoventral Mesoderm Patterning

During gastrulation, Xfrzb (A) is concentrated in the Spemann organizer, which is associated with specification of dorsal structures. Xwnt-8 (B) is excluded from this region, but is expressed in lateral and ventral mesoderm. In (C), the stylized (F) denotes the Frizzled-like amino-terminal sequence conserved between Frzb and Frizzled proteins (indicated by [Fz]), which are proposed as receptors for Wnt proteins. In the dorsal marginal zone, Xfrzb could compete with a Frizzled protein for Wnt binding and prevent signaling. In the ventral regions of the embryo, where Frzb is not present, Wnt-8 binding to the cognate receptor will not be affected. Question marks denote possible alternative actions of Frzb or Frzb–Wnt complexes.

body axes (Smith and Harland, 1991; Sokol et al., 1991). We interpreted the ability of Frzb to block this effect (Figures 3A–3C) as preliminary evidence that it may act as a functional Wnt antagonist. However, induction of secondary axes by overexpression of *Xwnt-8* prior to the midblastula transition may not reflect its function during gastrulation (Christian and Moon, 1993). We therefore tested the ability of Frzb to counteract effects of Xwnt-8 expressed under control of the cytoskeletal actin promotor, which is activated after the MBT. Frzb blocked post-MBT ventralization of animal cap explants by Xwnt-8 (Figure 3D) and rescued suppression of anterior structures in whole embryos injected dorsally with the CSKA-*Xwnt-8*. To provide additional evidence that the effects of Frzb can be attributed to inhibition of Wnt signaling directly, we demonstrated that Frzb could inhibit induction of *Siamois* and *Xnr3* by Xwnt-8. Recently, a dominant-negative Xwnt-8 has been shown to suppress somite formation and block expression of *MyoD* (Hoppler et al., 1996). Duplication of these actions by Frzb further confirms its ability to inhibit Wnt signaling (Figure 6). These lines of evidence provide strong support for the conclusion that Frzb can inhibit signaling by Xwnt-8.

The high degree of sequence similarity between the amino-terminal region of Frzb and the putative ligand binding domains of the Frizzled proteins would predict direct protein–protein interactions between Frzb and various Wnt proteins. The immunoprecipitation data presented in Figure 5 demonstrate that Frzb can indeed bind Wnt proteins directly. These results are complemented by experiments demonstrating that Frzb binds to cells transfected with a membrane-tethered Wnt-1 (Leyns et al., 1997). Since coimmunoprecipitation experiments with Frizzled and Wnt proteins have not been described, our results also further corroborate the identification of these proteins as Wnt receptors.

Our findings support the concept that Frzb counteracts Xwnt-8 signaling by direct extracellular binding. Its expression pattern suggests that under normal circumstances, Xfrzb may act to prevent Xwnt-8 from ventralizing mesoderm inappropriately in the Spemann organizer. This model is presented schematically in Figure 7. Precedent for a similar concept has been established by the observation that BMP-4, a key ventralizing factor in vertebrate development, can bind and be inactivated by two secreted factors, noggin (Zimmerman et al., 1996) and chordin (Piccolo et al., 1996). These proteins, like Frzb, are both expressed in the Spemann organizer, a

region of the developing Xenopus embryo destined to form dorsal tissues, and are thought to suppress the ventralizing activity of BMP-4. If chordin or noggin are overexpressed ventrally, within the normal expression domain of BMP-4, secondary dorsal axes are produced (Smith and Harland, 1992 Sasai et al., 1994). A similar effect is also observed with truncated receptors that block BMP signaling (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994) or with dominant-negative BMP ligands (Hawley et al., 1995; S. W. and M. M., unpublished data).

Since the Wnt proteins have been implicated in a wide variety of developmental processes, including limb patterning (Dealy et al., 1993; Parr et al., 1993; Parr and McMahon, 1995; Tickle, 1995) and certain neoplastic states, Frzb could modulate some of these processes as well. The amino-terminal region of Frzb that exhibits homology to the Frizzled proteins accounts for less than half of the molecule. The carboxyl-terminal region appears to be related to the netrins, which are chemotactic signals that influence axon guidance during development (Wadsworth and Hedgecock, 1996). Alternative actions for Frzb may thus be possible.

Conclusion

Frzb is a secretable protein expressed in the Spemann organizer, a region crucial to control of fundamental patterning events. It counteracts several actions of Xwnt-8. Frzb coimmunoprecipitates with Wnt proteins; this finding provides the first direct biochemical evidence for interaction between Wnts and other proteins. We propose that Frzb is a naturally occurring inhibitor of Wnt signaling that contributes to dorsoventral patterning of the mesoderm during vertebrate development.

Experimental Procedures

Isolation of *Xfrzb* cDNA

The primers 5′-TGGAACATGACTAAGATGCCC-3′ and 5′-CATATAC TGGCAGCTCCTCG-3′ were used for PCR labeling of a region of the bovine *frzb* cDNA sequence (Hoang et al., 1996) found to show a high degree of sequence identity to related genes from human and avian sources. Screening at low stringency (35°C in 20 mM Na_2HPO_4 [pH 7.2], 1 mM EDTA, 1% SDS) by standard procedures (Sambrook et al., 1989) allowed isolation of several clones from a stage 13 cDNA library (Richter et al., 1988); purified plaques were characterized by direct sequencing (Wang et al., 1995). Two closely similar clones were isolated, and one of these was chosen for further study.

Plasmids and Probes

The *Xfrzb* open reading frame was subcloned into pCR-Script (Stratagene) to generate probes for hybridization in situ. Both *Bfrzb* and *Xfrzb* were subcloned into pSP64R1 (Sergei Sokol, Harvard University) for mRNA injection experiments. The pSP64T-*Xwnt-8*^{myc} plasmid used for mRNA injections and translation in vitro and the CSKA-X8 expression plasmid (Christian and Moon, 1993) were provided by Dr. R. T. Moon (University of Washington, Seattle). A pGEM-5R-*Xwnt-8* plasmid (Smith and Harland, 1991) was used to generate probe for hybridization in situ. In vitro transcription was done using mMessage mMachine or MEGAscript kits from Ambion (Austin, TX). The plasmid p*LNCWnt1HA*, containing the open reading frame of mouse *Wnt1* and an HA tag near the C terminus, was kindly offered by Dr. Jan Kitajewski (Columbia University). The Xlmf25 plasmid used for hybridization in situ analysis of *MyoDa* (Scales et al., 1990) was the kind gift of Dr. Jon Scales (NICHD). The p*frzb* expression plasmid was described previously (Hoang et al., 1996). The prolactin plasmid (Amaya et al., 1991) was provided by Enrique Amaya.

Oocyte Injections

Enzymatically defolliculated oocytes were injected with 50 ng of mRNA and cultured with oocyte Ringer's solution (Kay, 1991). ^{35}S-methionine (500 μCi/mL) was added to the incubation medium for metabolic labeling studies.

Embryo Manipulations

Frogs and their embryos were maintained and manipulated using standard methods (Gurdon, 1967; Gurdon, 1977). All embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and Keller (Keller, 1991). mRNA injection experiments were performed by standard procedures, as described previously (Moos et al., 1995). Dorsal and ventral blastomeres were identified by size and pigment variations (Nieuwkoop and Faber, 1967). Lithium treatment was for 1 hr at 0.1 M (Kao and Elinson, 1988), and UV irradiation was done with a Stratalinker® (Smith and Harland, 1991). Animal cap explants were cultured in 0.7–1× Marc's Modified Ringer's solution (Kay, 1991). Activin was a gift from Michael Kuehn (National Cancer Institute), and bFGF was from Life Technologies.

Expression of Frzb and Wnt Proteins in COS7 Cells

COS7 cells (1.6×10^6 initial seeding density) were transfected with 5 μg of p*frzb* or p*LNCWnt1HA* or cotransfected with 4 μg of p*frzb* and pLNCWnt1HA in 100 mm dishes using 30 μl LipofectAMINE® (Life Technologies, Inc., Gaithersburg, MD). Transfections were carried out for 6 hr in serum-free Opti-MEM I® (Life Technologies). Thereafter, cells were incubated for 18 hr in media containing 10% fetal bovine serum. Subsequently, cells were cultured at 37°C for 24 hr in serum-free Opti-MEM I®. The cells were extracted for 30 min on ice with 50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% Deoxycholic acid, and 0.1% SDS and centrifuged at 12,000 × g for 5 min. The supernatants were saved for immunoprecipitation.

Immunoblotting and Immunoprecipitation

Embryos and oocytes were lysed by sonication on ice in 40 mM Tris base, 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride in a volume of 10 μl/embryo or oocyte. In some experiments, 20,000 × g supernatants were extracted with an equal volume of 1, 1, 2-trichlorotrifluoroethane (Evans and Kay, 1991). In vitro translations were performed in the presence of ^{35}S-methionine with nuclease-treated rabbit reticulocyte lysate and canine pancreatic microsomal membranes (Promega, Madison, WI) according to the instructions of the manufacturer. β-*lactamase* mRNA supplied with the kit was used as a positive control for translation and processing and as a negative control for nonspecific protein–protein interaction. SDS–PAGE was done with Novex 10% Nu-PAGE gels and the MOPS buffer system. Samples from embryos were precipitated with methanol/chloroform (Wessel and Flugge, 1984) prior to analysis. For metabolic labeling studies, gels were dried onto a single sheet of cellophane and imaged with BioMax MR2 film (Kodak) or a phosphor screen (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation was performed according to standard procedures (Harlow and Lane, 1988). Antiserum N374-PEP was prepared as described previously (Hoang et al., 1996); preliminary experiments confirmed that it was reactive against Xenopus Frzb. The clone 9E10 antibody (Boehringer) was used for precipitation or detection of the c-myc epitope, and hybridoma supernatant containing the 12CA5 monoclonal antibody was used for precipitation of the HA epitope. Immunoblot analyses of separated proteins were performed following transfer to nitrocellulose membranes, using 1:20,000 dilutions of primary antisera and 1:100,000 dilutions of peroxidase-conjugated secondary antibody. Bands were detected with the Super Signal Ultra peroxidase substrate (Pierce, Rockford, IL).

RT-PCR

Separate pools of embryos or explants were prepared from at least two different fertilizations for each condition reported. Total RNA was prepared with Trizol® and treated with Amplification Grade DNase (Life Technologies). Reverse transcription was done with

Superscript II (Life Technologies) as described by the manufacturer, with 1 μg of total RNA per reaction; 2% of the appropriate cDNA pool was used in each PCR. Amplification was performed in 10 μl reactions containing 50 mM TRIS·Cl (pH 8.3), 2 mM $MgCl_2$, 0.25% bovine albumin, 2.5% Ficoll 400, 5 mM tartrazine, 200 μM dNTPs, 1 μM each primer, and 0.2 U Taq polymerase (Boehringer Mannheim, Indianapolis, IN). Each cycle comprised 94°C, 0 seconds; 55°C, 0 seconds; 72°C, 40 seconds. A 1 min denaturation at 94°C preceded cycling, and a 2 min extension at 72°C was done at the end. An Idaho Technologies air thermal cycler was used in all experiments. Optimal cycle numbers were determined for each primer set by pilot experiments (Rupp and Weintraub, 1991; Niehrs et al., 1994). PCR products were separated on 2.5% agarose gels in TAE buffer, stained with SYBR Green 1® (Molecular Probes, Eugene, OR), and scanned with a Molecular Dynamics Fluorimager. PCR analysis was performed at least twice for each cDNA to confirm that the amplifications were reproducible. The primers for *Histone H4*, *MyoD*, *c-src*, *ADMP*, and *Xbra* were described previously (Niehrs et al., 1994; Hemmati-Brivanlou et al., 1994; Moos et al., 1995). The primers for *Siamois*, *Xnr3*, and *Xpo* have also been reported (Hoppler et al., 1996; Yang-Snyder et al., 1996). The *Xfrzb* primers for RT-PCR were F (5′-AGTAAGCCTACACATACAGGTTGG-3′) and R (5′-GCAGACTC CTCTGTCATATACGG-3′).

Hybridization In Situ

The procedures outlined by Harland (Harland, 1991) were followed, with modifications as described (Moos et al., 1995).

Histology

All embryos were embedded in JB-4 resin (Polysciences, Warrenton, PA). For conventional histological analysis, 1–3 μm sections were cut and stained with hematoxylin and eosin; 10–20 μM sections were taken from embryos stained by hybridization in situ.

Microscopy and Photography

Dark-field images of embryos were photographed with low angle oblique illumination and a Zeiss Stemi-6 dissecting microscope. Embryos cleared with benzyl alcohol/benzyl benzoate and the histological sections in Figure 1 were photographed under diascopic illumination with a Nikon FXA microscope. The sections in Figure 2A were photographed under multiple oblique illumination (Edge Scientific, Santa Monica, CA).

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